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(54) Title: CAMP FACTOR OF STREPTOCOCCUS UBERIS

(57) Abstract

The CAMP factor gen of *Streptococcus uberis* (*S. uberis*) is described, as well as the recombinant production of CAMP factor therefrom. CAMP factors can be used in vaccine compositions for the prevention and treatment of bacterial infections.

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CAMP FACTOR OF *STREPTOCOCCUS UBERIS*Technical Field

The present invention relates generally to bacterial antigens. More particularly, the present invention pertains to the recombinant production of CAMP factor from *Streptococcus uberis* (*S. uberis*) and the use of CAMP factors in vaccine compositions.

Background

S. uberis is an important cause of mastitis in dairy cattle and is responsible for about 20% of all clinical cases of mastitis (Bramley, A.J. and Dodd, F.H. (1984) *J. Dairy Res.* 51:481-512; Bramley, A.J. (1987) *Animal Health Nutrition* 42:12-16; Watts, J.L. (1988) *J. Dairy Sci.* 71:1616-1624). Since antimicrobial treatment is generally ineffective in treating *S. uberis* mastitis, the development of control measures must be based on an understanding of virulence factors and protective antigens involved in invasion and protection of the mammary gland (Collins et al. (1988) *J. Dairy Res.* 55:25-32; Leigh et al. (1990) *Res. Vet. Sci.* 49: 85-87; Marshall et al. (1986) *J. Dairy Res.* 53: 507-514).

It is known that some *S. uberis* strains can produce hyaluronic acid capsule (Hill, A.W. (1988) *Res. Vet. Sci.* 45:400-404), hyaluronidase (Schaufuss et al. (1989) *Zentralbl. Bakteriol. Ser. A* 271:46-53), R-like protein (Groschup, M.H. and Timoney, J.F. (1993) *Res. Vet. Sci.* 54:124-126), and a cohemolysin, the CAMP factor, also known as UBERIS factor (Skalka, B. and Smola, J. (1981) *Zentralbl. Bakteriol. Ser. A*

249:190-194). However, very little is known of their roles in pathogenicity.

The effect of CAMP factor was first described by Christie et al. in 1944 (Christie et al. 5 (1944) *Aus. J. Exp. Biol. Med. Sci.* 22:197-200). These authors found that group B streptococci (GBS), such as *S. agalactiae*, produced a distinct zone of complete hemolysis when grown near the diffusion zone of the *Staphylococcus aureus* beta-toxin, 10 sphingomyelinase. This phenomenon was called CAMP reaction and the compound for this reaction was characterized as the CAMP factor, an extracellular protein with a molecular weight of 23,500 (Bernheimer et al. (1979) *Infect. Immun.* 23:838-844). The CAMP 15 factor was subsequently purified from *S. agalactiae* and characterized as a 25,000 Da protein with a pI of 8.9 (Jürgens et al. (1985) *J. Chrom.* 348:363-370). The amino acid sequence of *S. agalactiae* CAMP factor was determined by Rühlmann et al. (Rühlmann et al. 20 (1988) *FEBS Lett.* 235:262-266).

The mechanism of the CAMP reaction has been described. See, e.g., Bernheimer et al. (1979) *Infect. Immun.* 23:838-844; Sterzik et al. "Interaction of the CAMP factor from *S. agalactiae* with artificial membranes." In: Alouf et al., eds. *Bacterial protein toxins*, London: Academic Press Inc, 1984; 195-196; 25 Sterzik et al. (1985) *Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. I Suppl.* 15:101-108; Fehrenbach et al. "Role of CAMP-factor (protein B) for virulence." In: Fehrenbach et al., eds. *Bacterial protein toxins*, Stuttgart: Gustav Fischer Verlag, 1988; 351-357; 30 Fehrenbach et al. "Interaction of amphiphilic bacterial polypeptides with artificial membranes." In: Alouf et al., eds. *Bacterial protein toxins*, London: Academic Press Inc., 1984:317-324.

CAMP factor has lytic action on a variety of target cells including sheep and bovine erythrocytes,

as well as on artificial membranes in which membrane phospholipids and sphingomyelin have been hydrolyzed by phospholipase or sphingomyelinase.

The role of CAMP factor in pathogenicity is unclear. A partially purified CAMP factor from *S. agalactiae* has been shown to be lethal to rabbits when injected intravenously (Skalka, B. and Smola, J. (1981) *Zentralbl. Bakteriol. Ser. A* 249:190-194). Furthermore, intraperitoneal injection of purified CAMP factor into mice has been shown to significantly raise the pathogenicity of a sublethal dose of group B streptococci (Fehrenbach et al. "Role of CAMP-factor (protein B) for virulence." In: Fehrenbach et al., eds. *Bacterial protein toxins*, Stuttgart: Gustav Fischer Verlag, 1988; 351-357). Additionally, like protein A of *S. aureus*, GBS CAMP factor can bind the Fc sites of immunoglobulins and has therefore been designated protein B (Jürgens et al. (1987) *J. Exp. Med.* 165:720-732).

In addition to GBS and *S. uberis*, other bacteria, including *Listeria monocytogenes* and *Listeria seeligeri* (Rocourt, J. and Grimont, P.A.D. (1983) *Int. J. Syst. Bacteriol.* 33:866-869) *Aeromonas* sp. (Figura, N. and Guglielmetti, P. (1987) *J. Clin. Microbiol.* 25:1341-1342), *Rhodococcus equi* (Fraser, G. (1961) *Nature* 189:246), and certain *Vibrio* spp. (Kohler, W. (1988) *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A* 270:35-40) produce reactions similar to the CAMP effect.

The CAMP factor genes of GBS and *A. pleuropneumoniae* have been cloned and expressed in *Escherichia coli* (Frey et al. (1989) *Infect. Immun.* 57:2050-2056; Schneewind et al. (1988) *Infect. Immun.* 56:2174-2179).

However, until now, the CAMP factor gene of *S. uberis* has not been cloned. Furthermore, the

protective capability of CAMP factor has not been previously studied.

Disclosure of the Invention

5 The present invention is based on the discovery of the CAMP factor gene of *S. uberis*, as well as the discovery that the CAMP factor is able to protect vertebrate subjects from infection. The CAMP factor, active immunogenic fragments thereof, active
10 analogs thereof, or chimeric proteins including the same, can be used, either alone or in combination with other antigens, in novel subunit vaccines to provide protection from bacterial infection in vertebrate subjects.

15 Accordingly, in one embodiment, the subject invention is directed to an isolated nucleic acid molecule comprising a coding sequence for an immunogenic

20 *Streptococcus uberis* CAMP factor. In additional embodiments, the invention is directed to recombinant vectors including the same, host cells transformed with these vectors and methods of recombinantly producing *S. uberis* CAMP factor.

25 In still further embodiments, the subject invention is directed to vaccine compositions comprising a pharmaceutically acceptable vehicle and an immunogenic CAMP factor. In particularly preferred embodiments, the CAMP factor is a *Streptococcus* CAMP factor.

30 In yet other embodiments, the present invention is directed to methods of treating or preventing bacterial infection, including streptococcal infections and mastitis, in a subject comprising administering to the subject a therapeutically effective amount of the above vaccine compositions.

In additional embodiments, the invention pertains to methods of producing vaccine compositions comprising (a) providing at least one immunogenic CAMP factor; and (b) combining the CAMP factor with a pharmaceutically acceptable vehicle.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

10 Brief Description of the Figures

Figure 1 depicts restriction enzyme maps of recombinant plasmid pJLD21 and subclones thereof, designated pJLD21-1 and pJLD21-2. Lines indicate *S. uberis* insert DNA, while boxes represent the multiple cloning sites of vector pTZ18R. The CAMP activities of recombinant plasmid pJLD21 and its derived subclones are indicated on the right (+, CAMP reaction positive; -, CAMP reaction negative). The small horizontal arrows represent start points and directions of sequencing experiments. The probe fragment used for Southern blot experiments is indicated by the large arrow. The bar at the bottom indicates the location of the open reading frame of CAMP factor gene of *S. uberis* (cfu).

25 Figure 2 shows the results of a CAMP reaction using *S. uberis* and recombinant *E. coli* clones. The CAMP reaction was done on a base #2 blood plate as described in the experimental section under Materials and Methods. Vertical streak: S, *Staphylococcus aureus*. Horizontal streak: 1, *S. uberis*; 2, *E. coli* JF1754(pTZ18R) (negative control); 3, *E. coli* JF1754(pJLD21) (CAMP-positive recombinant); 4, *E. coli* JF1754(pJLD21-2) (CAMP-positive subclone).

30 Figures 3A and 3B are representations of an SDS-PAGE and an immunoblot analysis, respectively, of *S. uberis* and *S. agalactiae* CAMP factors. Figure 3A shows a coomassie blue-stained 12% polyacrylamide-SDS

gel. Lane 1, partially purified *S. uberis* CAMP factor; lane 2, supernatant of *E. coli* JF1754(pJLD21) (CAMP positive); lane 3, supernatant of *E. coli* JF1754(pTZ18R) (CAMP negative); lane 4, 5 supernatant of *S. agalactiae*; lane MW, prestained molecular weight standards. Numbers on the left of the figure indicate the positions of the molecular weight markers (in thousands). Figure 3B shows an immunoblot of the gel presented in Figure 3A. Samples 10 were transferred to a nitrocellulose membrane and reacted with mouse antiserum against purified *S. uberis* CAMP factor.

Figures 4A-4C (SEQ ID NOS:____) show the nucleotide sequence of *S. uberis* CAMP factor gene and its promoter region. The start site of transcription is represented by +1, and the -10 and -35 regions of the promoter are indicated by the underlines. The putative Shine-Dalgarno sequence is shown as SD. The deduced amino acid sequence of *S. uberis* CAMP factor 20 is shown using the three-letter code below the nucleotide sequence. The signal peptide and mature peptide are specified.

Figure 5 shows a primer extension analysis of the promoter region of the *S. uberis* CAMP factor 25 gene. The primer extension reactions were done as described in the examples. The cellular RNAs used were obtained from *S. uberis* (lane 1), *E. coli* JF1754(pJLD21) (lane 2), and *E. coli* JF1754(pTZ18R) (lane 3). A dideoxy sequencing ladder was generated 30 by using the same primer and is shown as T, G, C, A above each lane.

Figure 6 (SEQ ID NOS:____) shows a comparison between *S. uberis* CAMP factor (upper lines, designated as "SUCAMP") and *S. agalactiae* CAMP factor (low lines, 35 designated as "SAGCAMP"). Amino acids are identified in the single-letter code. Identical amino acids are indicated by double dots (:). The aligned sequences

show 66.4% identity. Spaces (indicated by dash) were introduced in the sequences to optimize alignment.

Figure 7 shows a Southern blot analysis of different *S. uberis* strains. The origin of the probe used is indicated in Figure 1; chromosomal DNA from each *S. uberis* strain was cut with *Hind*III. Lanes 1, 2, 3 and 4 are the DNA samples from ATCC strains 9927, 13386, 13387 and 19436, respectively; lanes 5, 6, 7 and 8 are the DNA samples from field isolates. The arrowhead on the right indicates the position and size of the hybridizing restriction enzyme fragments. CAMP reaction phenotype of each strain is indicated at the bottom by "+" (CAMP reaction positive) and "-" (CAMP reaction negative).

Figure 8 depicts the construction of pGH-CAMP. The CAMP reactions for each clone from which the plasmid was derived are shown to the right of the figure; + indicates a positive reaction and - a negative reaction. Open boxes indicate pTZ18R and hatched boxes indicate pGH433.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.K. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL press, 1986); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S.

Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

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A. Definitions

In describing the present invention, the following terms will be employed, and are intended to 10 be defined as indicated below.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, 15 for example, reference to "a CAMP factor" includes a mixture of two or more CAMP factors, and the like.

The term "CAMP factor" or a nucleotide sequence encoding the same, intends a protein or a nucleotide sequence, respectively, which is derived 20 from a CAMP factor gene found in a variety of bacterial species, including, without limitation *Streptococcus uberis*, group B streptococci (GBS) such as *S. agalactiae* (Jürgens et al. (1985) *J. Chromatogr.* 25 348:363-370; and Rühlmann et al. (1988) *FEBS Lett* 235:262-266), *Listeria monocytogenes* and *Listeria seeligeri* (Rocourt, J. and Grimont, P.A.D. (1983) *Int. J. Syst. Bacteriol.* 33:866-869), *Aeromonas* sp. (Figura, N. and Guglielmetti, P. (1987) *J. Clin. Microbiol.* 25:1341-1342), *Rhodococcus equi* (Fraser, G. (1961) *Nature* 189:246), and certain *Vibrio* spp. (Kohler, W. (1988) *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A* 270:35-40).

A representative CAMP factor gene, derived 35 from *S. uberis*, is found in plasmid pJLD21 and depicted in Figures 4A-4C (SEQ ID NOS: ____).

The derived protein or nucleotid sequences need not be physically derived from the gene described above, but may be generated in any manner, including for example, chemical synthesis, isolation (e.g., from an organism that produces the CAMP factor) or by recombinant production, based on the information provided herein. Furthermore, the term intends proteins having amino acid sequences substantially homologous to contiguous amino acid sequences encoded by the genes, which display immunological activity. Thus, the terms include full-length, as well as immunogenic and truncated and partial sequences, as well as active analogs and precursor forms of the proteins, such as those forms including the signal sequence, described more fully below.

The terms also include proteins in neutral form or in the form of basic or acid addition salts depending on the mode of preparation. Such acid addition salts may involve free amino groups and basic salts may be formed with free carboxyls. Pharmaceutically acceptable basic and acid addition salts are discussed further below. In addition, the proteins may be modified by combination with other biological materials such as lipids (both those occurring naturally with the molecule or other lipids that do not destroy immunological activity) and saccharides, or by side chain modification, such as acetylation of amino groups, phosphorylation of hydroxyl side chains, oxidation of sulphhydryl groups, glycosylation of amino acid residues, as well as other modifications of the encoded primary sequence.

The term therefore intends deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined above. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those

substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; 5 (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cystine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as 10 aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, or vice versa; an aspartate with a glutamate or vice versa; a threonine with a serine or vice versa; or a similar conservative 15 replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule, but possessing minor amino acid 20 substitutions that do not substantially affect the immunogenicity of the protein, are therefore within the definition of the reference polypeptide.

The term "streptococcal CAMP factor" intends a CAMP factor, as defined above, derived from a 25 streptococcal species that produces the same, including *S. uberis* and GBS such as *S. agalactiae*. An "*S. uberis* CAMP factor" is a CAMP factor as defined above, derived from *S. uberis*.

By "mastitis" is meant an inflammation of 30 the mammary gland in mammals, including in cows, ewes, goats, sows, mares, and the like, caused by various bacteria that produce CAMP factors, described more fully below. The infection manifests itself by the infiltration of phagocytic cells in the gland. 35 Generally, 4 clinical types of mastitis are recognized: (1) peracute, associated with swelling, heat, pain, and abnormal secretion in the gland and

accompanied by fever and other signs of systemic disturbance, such as marked depression, rapid weak pulse, sunken eyes, weakness and complete anorexia; (2) acute, with changes in the gland similar to those above but where fever, anorexia and depression are slight to moderate; (3) subacute, where no systemic changes are displayed and the changes in the gland and its secretion are less marked; and (4) subclinical, where the inflammatory reaction is detectable only by standard tests for mastitis.

Standard tests for the detection of mastitis include but are not limited to, the California Mastitis Test, the Wisconsin Mastitis Test, the Nagase test, the electronic cell count and somatic cell counts used to detect a persistently high white blood cell content in milk. In general, a somatic cell count of about 300,000 to about 500,000 cells per ml or higher, in milk will indicate the presence of infection. Thus, a vaccine is considered effective in the treatment and/or prevention of mastitis when, for example, the somatic cell count in milk is retained below about 500,000 cells per ml. For a discussion of mastitis and the diagnosis thereof, see, e.g., *The Merck Veterinary Manual. A Handbook of Diagnosis, Therapy, and Disease Prevention and Control for the Veterinarian*, Merck and Co., Rahway, New Jersey, 1991.

An "isolated" nucleic acid molecule is a nucleic acid molecule separate and discrete from the whole organism with which the molecule is found in nature; or a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences (as defined below) in association therewith.

The term "epitope" refers to the site on an antigen or hapten to which specific B cells and T

cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the 5 ability of one antibody to block the binding of another antibody to a target antigen.

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to 10 the composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ 15 T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display a protective immunological response to the CAMP factor in question, e.g., the host will be protected from 20 subsequent infection by the pathogen and such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host or a quicker recovery time.

The terms "immunogenic" protein or 25 polypeptide refer to an amino acid sequence which elicits an immunological response as described above. An "immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of the CAMP factor in question, including the precursor and mature 30 forms, analogs thereof, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of a CAMP factor which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified by, 35 e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the

peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) Proc. Natl. Acad. Sci. USA 81:3998-4002; Geysen et al. (1986) Molec. Immunol. 23:709-715.

10 Immunogenic fragments, for purposes of the present invention, will usually be at least about 2 amino acids in length, more preferably about 5 amino acids in length, and most preferably at least about 10 to 15 amino acids in length. There is no critical upper limit to the length of the fragment, which could comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or 15 more epitopes of the CAMP factor or factors in question.

"Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous 20 DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be 25 attached so as to bring about the replication of the attached segment.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a 30 polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory elements. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. 35 A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g.,

mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

DNA "control elements" refers collectively 5 to promoters, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not 10 all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

"Operably linked" refers to an arrangement 15 of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they 20 function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter and the coding sequence and the promoter can still be considered "operably linked" to the coding sequence.

Similarly, a coding sequence is "operably 25 linked to" another coding sequence (i.e., in the case of a chimeric protein) when RNA polymerase will transcribe the two coding sequences into mRNA, which is then translated into the polypeptides encoded by 30 the two coding sequences. The coding sequences need not be contiguous to one another so long as the transcribed sequence is ultimately processed to produce the desired protein.

A control element, such as a promoter, 35 directs the transcription of a coding sequence in a cell when RNA polymerase will bind the promoter and transcribe the coding sequence into mRNA, which is

then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an 5 exogenous nucleic acid molecule.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA 10 making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become 15 integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing 20 the exogenous DNA.

"Homology" refers to the percent of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by 25 techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs such as ALIGN, Dayhoff, 30 M.O. (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3, National biomedical Research Foundation, Washington, DC.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions 35 which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested

fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when at least about 80%, preferably at least about 90%, and most preferably at least about 95% of the nucleotides or 5 amino acids match over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing identity to the specified DNA or polypeptide sequence. DNA sequences that are 10 substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook 15 et al., *supra*; *DNA Cloning*, vols I & II, *supra*; *Nucleic Acid Hybridization*, *supra*.

The term "functionally equivalent" intends 20 that the amino acid sequence of the CAMP factor is one that will elicit a substantially equivalent or enhanced immunological response, as defined above, as compared to the response elicited by a CAMP factor having identity with either the mature sequence for the reference CAMP factor, or an immunogenic portion thereof.

A "heterologous" region of a DNA construct 25 is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, 30 the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence 35 itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring

mutational events do not give rise to a heterologous region of DNA, as used herein.

The term "treatment" as used herein refers to either (i) the prevention of infection or
5 reinfection (prophylaxis), or (ii) the reduction or, elimination of symptoms of the disease of interest (therapy).

By "vertebrate subject" is meant any member of the subphylum chordata, including, without
10 limitation, mammals such as cattle, sheep, pigs, goats, horses, and humans; domestic animals such as dogs and cats; and birds, including domestic, wild and game birds such as cocks and hens including chickens, turkeys and other gallinaceous birds; and fish. The
15 term does not denote a particular age. Thus, both adult and newborn animals, as well as fetuses, are intended to be covered.

B. General Methods

Central to the present invention is the discovery that the CAMP factor is capable of eliciting a protective immune response in a vertebrate subject. The gene for the *S. uberis* CAMP factor has been isolated and characterized and the CAMP factor encoded thereby sequenced. The protein product from the *S. uberis* CAMP factor gene has been shown to protect cattle from subsequent challenge with *S. uberis*.

In particular, the complete DNA sequence of *S. uberis* CAMP factor is shown in Figures 4A-4C (SEQ ID NO: ____). A major transcript of the CAMP factor gene is found beginning with an "A" residue (depicted at the +1 position in Figure 4A). -10 and -35 regions, characteristic of *E. coli* promoters (Harley, C.B. and Reynolds, R.P. (1987) *Nucleic Acids Res.* 35:2343-2361), are found upstream of the transcriptional start site, as indicated in Figure 4A. An open reading frame beginning with an ATG codon is

located at positions 157 to 159 and terminates with a TAA stop codon at positions 925 to 927. The ATG start codon is preceded by the purine-rich sequence AAGAGG, which serves as a ribosome binding site in *E. coli*

5 (Stormo et al. (1982) *Nucleic Acids Res.* **10**:2971-2996).

As shown in Figures 4A-4C, the *S. uberis* CAMP factor gene encodes a preprotein of about 256 amino acids (amino acid residues 1 through 256, inclusive, of Figures 4A-4C) that includes an N-terminal signal sequence approximately 28 amino acids in length. The precursor molecule has a calculated molecular weight of 28,363 Da. The mature *S. uberis* CAMP factor thus includes amino acid residues 29 through 256, inclusive, as depicted in Figures 4A-4C. As discussed further below, the portion of the CAMP factor gene encoding the signal sequence can be included in constructs that encode the CAMP factor to direct secretion of the CAMP factor upon expression.

10 15 Additionally, the CAMP factor signal sequence and the nucleic acid sequence encoding the same can be used with heterologous proteins and nucleic acid molecules, to aid in the secretion thereof.

As shown in Figure 6 (SEQ ID NOS:____), alignment of the 226-amino acid sequence of the *S. agalactiae* CAMP factor with the deduced 256 amino acids of the *S. uberis* CAMP factor shows that 66.4% of the amino acid residues are identical. Additionally, antibodies raised against purified *S. uberis* CAMP factor cross-react with *S. agalactiae* protein B. As shown in the examples, the *S. uberis* CAMP factor is secreted when produced recombinantly in *E. coli*.

The exact localization and sequence of the CAMP factor gene allows for *in vitro* mutagenesis studies to assess the functions of different domains on the CAMP protein. Also, the present data permits the generation of stable CAMP factor-synthesis

deficient mutants through gene replacement and other molecular techniques. Comparison of the virulence of native and mutant *S. uberis* strains in animals provides important information regarding the 5 contribution of the CAMP factor to the pathogenicity of bacteria expressing CAMP factors.

The CAMP factors, immunogenic fragments thereof or chimeric proteins including the same, can be provided in subunit vaccine compositions. In addition to use in vaccine compositions, the proteins or antibodies thereto can be used as diagnostic reagents to detect the presence of infection in a vertebrate subject. Similarly, the genes encoding the proteins can be cloned and used to design probes to detect and 15 isolate homologous genes in other bacterial strains.

The vaccine compositions of the present invention can be used to treat or prevent a wide variety of bacterial infections in vertebrate subjects. For example, vaccine compositions including 20 CAMP factors from *S. uberis* and/or group B streptococci (GBS), such as *S. agalactiae*, can be used to treat streptococcal infections in vertebrate subjects that are caused by these species. In particular, *S. uberis* and *S. agalactiae* are common 25 bacterial pathogens associated with mastitis in bovine, equine, ovine and goat species. Additionally, group B streptococci, such as *S. agalactiae*, are known to cause numerous other infections in vertebrates, including septicemia, meningitis, bacteremia, 30 impetigo, arthritis, urinary tract infections, abscesses, spontaneous abortion etc. Hence, vaccine compositions containing streptococcal CAMP factors will find use in treating and/or preventing a wide variety of streptococcal infections.

35 Similarly, CAMP factors derived from *Listeria monocytogenes* and *L. seeligeri*, *Aeromonas* sp., *Rhodococcus equi*, and *Vibrio* spp. will find use

for treating bacterial infections caused by these species. For example, CAMP factors can be used to prevent or treat listeriosis in a wide range of animals and birds, including humans. The infection 5 can manifest itself as encephalitis and meningoencephalitis in ruminants and avian species such as geese, chickens, turkeys, ducks, canaries and parrots; septicemia in monogastric animals, neonatal ruminants and poultry; and spontaneous abortion and 10 latent infections in a wide variety of animals. *Aeromonas* causes infections in fish, including in salmonids, aquarium fish, goldfish, freshwater and marine fish; as well as infections in caged birds and in amphibians and reptiles. *Rhodococcus equi* causes 15 respiratory infections, lymphangitis, peritonitis, enteritis, abscesses and spontaneous abortion in horses. *Vibrio* causes vibriosis in many cultured, aquarium and wild marine and estuarine fish; infections of open wounds in cetaceans; and avian 20 vibrionic hepatitis and avian infectious hepatitis in chickens.

Thus, it is readily apparent that CAMP factor vaccines can be used to treat and/or prevent a wide variety of bacterial infections in numerous 25 species.

CAMP factors from various species can be used either alone or in combination in the vaccine compositions of the present invention. For example, it will sometimes be preferable to have more than one 30 epitope of one or more of the CAMP factors in the vaccine compositions of the present invention so that the subject in question can be provided with a broad spectrum of protection against infection. In its simplest form, this can be achieved by employing a 35 polypeptide encoding the complete sequence of one of the CAMP factors, or by employing a combination of polypeptides encoding the sequences of two or more of

the CAMP factors or epitopes of the CAMP factors. Thus, the vaccine compositions could comprise, for example various combinations of one or more of the CAMP factors, or a combination of several of the CAMP factors, or even several epitopes derived from the CAMP factors.

Furthermore, the vaccine compositions of the present invention can include other bacterial, fungal, viral or protozoal antigens. These antigens can be provided separately or even as fusion proteins comprising fragments of one or more of the CAMP factors fused to these antigens.

Production of the CAMP Factors

The above described CAMP factors and active fragments, analogs and chimeric proteins derived from the same, can be produced by a variety of methods. Specifically, the CAMP factors can be isolated directly from bacteria which express the same. This is generally accomplished by first preparing a crude extract which lacks cellular components and several extraneous proteins. The desired proteins can then be further purified i.e. by column chromatography, HPLC, immunoabsorbent techniques or other conventional methods well known in the art.

More particularly, techniques for isolating CAMP factors have been described in e.g., Skalka, B. and Smola, J. (1981) *Zbl. Bakt. Hyg., I. Abt. Orig.* A249:190-194; Skalka et al. (1980) *Zbl. Vet. Med.* B27:559-566; Skalka et al. (1979) *Zbl. Vet. Med.* B26:679-687; Bernheimer et al. (1979) *Infect. Immun.* 23:838-844; Jürgens et al. (1985) *J. Chrom.* 348:363-370; Jürgens et al. (1987) *J. Exp. Med.* 165:720-732.

Alternatively, the proteins can be recombinantly produced as described herein. As explained above, these recombinant products can take the form of partial protein sequences,

- full-length sequences, precursor forms that include signal sequences, mature forms without signals, or even fusion proteins (e.g., with an appropriate leader for the recombinant host, or with another subunit
- 5 antigen sequence for *Streptococcus* or another pathogen).

The CAMP factor genes of the present invention can be isolated based on the ability of the protein products to display CAMP activity, using CAMP assays as described below. Thus, gene libraries can be constructed and the resulting clones used to transform an appropriate host cell. Colonies can be pooled and screened for clones having CAMP activity. Colonies can also be screened using polyclonal serum 10 or monoclonal antibodies to the desired antigen.

Alternatively, once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen DNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., *DNA Cloning*: 20 Vol. I, *supra*; *Nucleic Acid Hybridization*, *supra*; *Oligonucleotide Synthesis*, *supra*; Sambrook et al., *supra*. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA 25 sequencing that the particular library insert contains the desired CAMP factor gene or a homolog thereof.

Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequences can be designed 30 with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be

used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, generally, *DNA Cloning: Vols. I & II, supra*; Sambrook et al., *supra*; B. Perbal, *supra*.

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. If signal sequences are included, they can either be the native, homologous sequences, or heterologous sequences. For example, the signal sequence for *S. uberis* CAMP factor (shown in Figure 4A), can be used for secretion of various

CAMP factors, as can a number of other signal sequences, well known in the art. Leader sequences can be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 5 4,425,437; 4,338,397.

Other regulatory sequences may also be desirable which allow for regulation of expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present 10 in the vector, for example, enhancer sequences. 15

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding 20 sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the 25 control sequences with the appropriate orientation; i.e., to maintain the proper reading frame. It may also be desirable to produce mutants or analogs of the CAMP factor of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence 30 encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are described in, e.g., Sambrook et al., 35 *supra*; *DNA Cloning*, Vols. I and II, *supra*; *Nucleic Acid Hybridization*, *supra*.

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the proteins of the present invention are produced by culturing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into the growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The proteins of the present invention may also be produced by chemical synthesis such as solid

phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

The CAMP factors, fragments, analogs and chimeras containing the same, can be tested for CAMP activity using any of several standard tests. For example, CAMP factors are known to display lytic action on a variety of target cells including bovine and ovine erythrocytes. Thus, a convenient method for testing for CAMP factor activity utilizes standard hemolytic reactions using ovine or bovine erythrocytes. See, e.g., Christie et al. (1944) *Aus. J. Exp. Biol. Med. Sci.* 22:197-200; Brown et al. (1974) *Infect. Immun.* 9:377-383; Darling, C.L. (1975) *J. Clin. Microbiol.* 1:171; Wilkinsin, H.W. (1977) *J. Clin. Microbiol.* 6:42; Bernheimer et al. (1979) *Infect. Immun.* 23:838-844; Skalka, B. and Smola, J. (1981) *Zbl. Bakt. Hyg., I. Abt. Orig.* A249:190-194; Huser et al. (1983) *J. Gen. Microbiol.* 129:1295.

Activity can also be tested by monitoring the release of entrapped marker molecules from liposomes made from materials susceptible to disruption by CAMP factors. For example, CAMP activity can be monitored using [¹⁴C]glucose-containing liposomes prepared from, e.g., sphingomyelin, cholesterol and dicetyl phosphate, and measuring the release of trapped [¹⁴C]glucose due to disruption of the liposomes by the CAMP factor. See, e.g., Bernheimer et al. (1979) *Infect. Immun.* 23:838-844. Similarly, ATP release from liposomes in the presence of CAMP factor can be monitored as described in Sterzik et al. (1984) "Interaction of the CAMP factor from *S. agalactiae* with artificial membranes" In:

Alouf et al., eds. *Bacterial protein toxins*, London: Academic Press Inc., 1984:195-196; and Sterzik et al. (1985) *Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Suppl.* 15:101-108. See, also Fehrenbach et al. (1984) 5 "Interaction of amphiphilic bacterial polypeptides with artificial membranes." In: Alouf et al., eds. *Bacterial protein toxins*, London: Academic Press Inc., 1984:317-324.

The CAMP factors of the present invention or 10 their fragments can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an antigen of the present invention, or its fragment, or 15 a mutated antigen. Serum from the immunized animal is collected and treated according to known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by 20 immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the CAMP factors and to the fragments thereof, can also be readily produced by one skilled in the art. The general 25 methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or 30 transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling et al., *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 35 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the

CAMP factor of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against. Both polyclonal and monoclonal antibodies can also be used for passive immunization or can be combined with subunit vaccine preparations to enhance the immune response.

10

Vaccine Formulations and Administration

The CAMP factors of the present invention can be formulated into vaccine compositions, either alone or in combination with other antigens, for use in immunizing subjects as described below. Methods of preparing such formulations are described in, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18 Edition, 1990. Typically, the vaccines of the present invention are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in or suspension in liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is generally mixed with a compatible pharmaceutical vehicle, such as, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents and pH buffering agents.

Adjuvants which enhance the effectiveness of the vaccine may also be added to the formulation. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, dimethyldioctadecyl ammonium bromide (DDA), oils, oil-in-water emulsions,

saponins, cytokines, and other substances known in the art.

The CAMP factor may be linked to a carrier in order to increase the immunogenicity thereof.

5 Suitable carriers include large, slowly metabolized macromolecules such as proteins, including serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art;

10 polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles.

15 The CAMP factors may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by,

20 for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

25 Other suitable carriers for the CAMP factors of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651. Also useful is a fusion product of a viral protein and the subject immunogens made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state.

30 Alternatively, the proteins of the present invention may be coupled to erythrocytes, preferably the

subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

Furthermore, the CAMP factors (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Injectable vaccine formulations will contain a "therapeutically effective amount" of the active ingredient, that is, an amount capable of eliciting an immune response in a subject to which the composition is administered. In the treatment and prevention of mastitis, for example, a "therapeutically effective amount" would preferably be an amount which controls infection, as measured by, e.g. the ability of the composition to retain or bring the somatic cell count in milk below about 500,000 cells per ml. The exact amount is readily determined by one skilled in the art using standard tests. The CAMP factor will typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. With the present vaccine formulations, 20 to 500 µg of active ingredient per ml of injected solution should be adequate to raise an immunological response when a dose of 1 to 3 ml per animal is administered.

To immunize a subject, the vaccine is generally administered parenterally, usually by intramuscular injection. Other modes of administration, however, such as subcutaneous, 5 intraperitoneal and intravenous injection, are also acceptable. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. Effective dosages 10 can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the vaccine in at least one dose, and preferably two doses. Moreover, the animal may be 15 administered as many doses as is required to maintain a state of immunity to infection.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, 20 oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing 25 the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium 30 saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active 35 ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal

mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives 5 such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations 10 are made by incorporating the protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and 15 certain polyacids or polyesters such as those used to make resorbable sutures. The CAMP factors can also be delivered using implanted mini-pumps, well known in the art.

The CAMP factors of the instant invention 20 can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, 25 vaccinia virus recombinants expressing the novel proteins can be constructed as follows. The DNA encoding the particular protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, 30 such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant protein into the 35 viral genome. The resulting TK-recombinant can be selected by culturing the cells in the presence of 5-

bromodeoxyuridine and picking viral plaques resistant thereto.

An alternative route of administration involves gene therapy or nucleic acid immunization.

5 Thus, nucleotide sequences (and accompanying regulatory elements) encoding the subject CAMP factors can be administered directly to a subject for *in vivo* translation thereof. Alternatively, gene transfer can be accomplished by transfecting the subject's cells or
10 tissues *ex vivo* and reintroducing the transformed material into the host. DNA can be directly introduced into the host organism, i.e., by injection (see International Publication No. WO/90/11092; and Wolff et al. (1990) *Science* 247:1465-1468). Liposome-
15 mediated gene transfer can also be accomplished using known methods. See, e.g., Hazinski et al. (1991) *Am. J. Respir. Cell Mol. Biol.* 4:206-209; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278-281; Canonico et al. (1991) *Clin. Res.* 39:219A; and Nabel et al. (1990)
20 *Science* 1990) 249:1285-1288. Targeting agents, such as antibodies directed against surface antigens expressed on specific cell types, can be covalently conjugated to the liposomal surface so that the nucleic acid can be delivered to specific tissues and
25 cells susceptible to infection.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

C. Experimental

Materials and Methods

35 Enzymes were purchased from commercial sources, and used according to the manufacturers'

directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

- In the isolation of DNA fragments, except where noted, all DNA manipulations were done according 5 to standard procedures. See, Sambrook et al., *supra*. Restriction enzymes, T₄ DNA ligase, *E. coli*, DNA polymerase I, Klenow fragment, and other biological reagents can be purchased from commercial suppliers and used according to the manufacturers' directions. 10 Double stranded DNA fragments were separated on agarose gels.

Bacterial strains, plasmids and growth conditions:

- The *E. coli* strain used for cloning the *S. 15 uberis* CAMP factor gene was JF1754 (*hsdR lac gal metB leuB hisB*) (McNeil, J.B. and Friesen, J.D. (1981) *Mol. Gen. Genet.* 184:386-393). Competent *E. coli* JF1754 was made as previously described (Hanahan, D. "Techniques for transformation of *E. coli*." In: Glover 20 DM, ed. *DNA cloning (Volume I): a practical approach*. Oxford: IRL Press, 1985:109-135). *E. coli* cells were grown in Luria broth (Difco Laboratories) or on Luria-agar (Difco Laboratories) plates. Ampicillin was used at 50 µg/ml for the growth of *E. coli* strains 25 containing recombinant plasmids. Four *S. uberis* strains, as well as *S. agalactiae* and *S. aureus*, were obtained from the American Type Culture Collection (ATCC Accession Nos. 9927, 13386, 13387, 19436, 27541 and 25923, respectively). Other *S. uberis* strains are 30 field isolates kindly provided by M. Chirino-Trejo, University of Saskatchewan. All streptococcal strains were grown in brain heart infusion broth (BHI, Difco Laboratories) or on base #2 blood agar plates with 5% sheep blood (PML microbiologicals).
- 35 The cloning vector pTZ18R (Mead et al. (1986) *Protein Eng.* 1:67-74) was obtained from Pharmacia Canada Ltd.

Preparation of S. aureus beta-toxin:

S. aureus was cultured in BHI for 18 h at 37°C and the supernatant obtained after centrifugation at 5,000 g was sterilized by filtration through a 5 0.22-µM filter (Nalge company). This material, referred to as crude beta-toxin, was stored at -20°C.

CAMP reaction

Bacteria were screened for CAMP activity as 10 described (Schneewind et al. (1988) *Infect. Immun.* 56:2174-2179). Briefly, strains were streaked perpendicular to a streak of beta-toxin-producing *S. aureus* on blood agar plates and after 6 h - 20 h 15 incubation at 37°C, they were observed for hemolysis.

Purification of CAMP factor

CAMP factor was partially purified from the culture supernatant of *S. uberis* (ATCC Accession No. 9927) by Octyl-Sepharose CL-4B (Pharmacia) 20 chromatography as described by Jürgens et al. (1985) *J. Chrom.* 348:363-370.

Polyclonal antibodies

To analyse the recombinant CAMP factor of *S. 25 uberis*, polyclonal antibodies directed against the purified CAMP factor were obtained. Mice were immunized by intraperitoneal injection of 20 µg of the purified CAMP protein with complete Freund adjuvant. This primary immunization was followed 3 weeks later 30 by the second intraperitoneal injection of the same amount of CAMP protein with incomplete Freund adjuvant and another 3 weeks later by the third intravenous injection of 20 µg of CAMP protein with incomplete Freund adjuvant. The blood serum samples were then 35 taken 10 days later.

PAGE and immunoblotting

Protein samples of *S. agalactiae* and *E. coli* were obtained from culture supernatants by trichloroacetic acid (TCA)-precipitation at a final concentration of 10%. SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed as described by Laemmli (Laemmli, U.K. (1970) *Nature* 227:680-685). Proteins were electroblotted onto nitrocellulose membranes as recommended by the supplier (Bio-Rad) and the blots were developed as described elsewhere (Theisen, M. and Potter, A.A. (1992) *J. Bacteriol.* 174:17-23) with the following differences. The first antiserum used was mouse polyclonal antiserum against partially-purified *S. uberis* CAMP protein, and it was absorbed with antigens of the *E. coli* host strain as described previously (Frey et al. (1989) *Infect. Immun.* 57:2050-2056). The second antibody used in blotting procedure was the goat anti-mouse IgG coupled to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.).

DNA manipulations

All molecular techniques were as recommended by the supplier (Pharmacia Canada Ltd.) or Sambrook et al., supra. Chromosomal DNA of *S. uberis* was prepared from cells grown in 100 ml BHI plus 5% (w/v) glycine. Cells were pelleted and resuspended in 2.5 ml of TES buffer (30 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl; pH 8.0) with 25% sucrose and 1.6 mg/ml lysozyme (Sigma). The suspension was incubated for 1 h at 37°C, followed by freezing at -70°C. The frozen cells were thawed in a 65°C water bath. EDTA and proteinase K (Pharmacia) were added to final concentrations of 20 mM and 1.2 mg/ml, respectively, before incubation at 65°C for 30 min. To lyse cells completely, sarkosyl was added to 1% and incubated at 37°C for 1 h. Two ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.9) was

added prior to phenol: chloroform extraction. DNA was recovered by ethanol precipitation and was treated with RNase (Pharmacia Canada Ltd.).

5 Size-fractionated Sau3AI-digested chromosomal DNA fragments were isolated by sucrose density gradient centrifugation (Sambrook et al., supra).

10 DNA sequence was determined by the dideoxy-chain termination method of Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467 on double-stranded plasmid templates by using a ^{T7} Sequencing kit (Pharmacia Canada Ltd.).

RNA analyses

15 RNA from *E. coli* strains was isolated as described previously (Lloubes et al. (1986) Nucleic Acid Res. 14:2621-2636) with an additional RNase-free DNase I digestion. RNA from *S. uberis* was prepared as follows. The cell pellet from a 10 ml culture 20 ($OD_{600}=0.6$) was resuspended in 250 μ l of TE buffer (pH 8.0) containing 500 u of mutanolysin (Sigma) and incubated at 37°C for 30 min. Lysis buffer (250 μ l) (60 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM EDTA, 2% SDS) and 100 μ g/ml (final concentration) of proteinase K was added and the incubation continued for 1 h. The sample was extracted once with 65°C phenol (water saturated, pH 4.0) and twice with room temperature phenol. RNA was recovered by ethanol precipitation 25 and treated with DNase I (Pharmacia Canada Ltd.).

30 Primer extension assay was performed as described by Miller et al. (1986) Nucleic Acids Res. 14:7341-60. RNasin and moloney murine leukemia virus reverse transcriptase were obtained from Pharmacia Canada Ltd.

35

Example 1Cloning and expression of the
S. uberis CAMP factor gene

Chromosomal DNA of *S. uberis* (ATCC 9927) was
5 partially digested with *Sau*3AI and size fractionated
in a sucrose gradient; from this, 2- to 5-kb DNA
fragments were recovered. The ends of these fragments
were partially filled in with dGTP and dATP and
ligated into pTZ18R which was cut with *Sal*I and
10 partially filled in with dTTP and dCTP. Following
transformation of *E. coli* JF1754 competent cells,
clones expressing the CAMP factor gene were identified
on blood plates with ampicillin and beta-toxin on the
surface. Six clones from a total of 10,000 were
15 phenotypically hemolytic and each one mediated a
distinct CAMP reaction. One of them, containing
recombinant plasmid pJLD21, was selected for further
study and its CAMP reaction is shown in Figures 1 and
2.

20 Plasmid pJLD21 contained a 5.2 kb insert
fragment and the CAMP factor gene, *cfu*, was localized
within a 3.2 kb *Bam*HI fragment after the CAMP-positive
subclone pJLD21-2 was generated (Figures 1 and 2).
This subclone was further analysed with more
25 restriction enzymes for sequencing purposes.

To study the expression of the recombinant
CAMP factor, SDS-PAGE analysis of supernatant proteins
from *Cfu*⁺ *E. coli* JF1754(pJLD21) and host *E. coli*
JF1754 (pTZ18R) was performed (Figure 3A). Compared
30 to the vector control, no distinguishable band was
observed in the lane containing supernatant from the
Cfu⁺ clone, indicating that either expression was at a
very low level or the protein was not secreted
efficiently. To identify the CAMP factor encoded by
35 pJLD21, the proteins separated by SDS-PAGE were
transferred to a nitrocellulose membrane and
immunoblotted (Figure 4B). The *Cfu*⁺ *E. coli* clone

carrying pJLD21 expressed a protein with molecular weight of 28,000 (lane 2), similar to the native CAMP factor of *S. uberis* (lane 1).

Another expression plasmid for the *S. uberis* CAMP factor, pGH-CAMP, was constructed as shown in Figure 8. In particular, A 1.7 kb EcoRI-BamHI fragment of pJLD21-2 was filled in with Klenow polymerase and inserted into pGH433 which was cut by BamHI and filled in a similar fashion. Plasmid pGH433 is an expression vector containing a tac promoter, a translational start site with restriction enzyme sites allowing ligation in all three reading frames followed by stop codons in all reading frames. See, Theisen, M. and Potter, A.A. (1992) *J. Bacteriol.* 174:17-23.

The expression plasmids were used to transform *E. coli* JF1754 (described above). The CAMP factor was prepared from inclusion bodies as described in, e.g., Rossi-Campos et al. (1992) *Vaccine* 10:512-518, for use in the vaccine trials below. Briefly, bacteria were grown to mid-log phase and isopropyl- β ,D-thiogalactoside (IPTG) was added and the cultures were incubated with vigorous agitation at 37°C. The bacteria were harvested by centrifugation, resuspended and frozen at -70°C. The frozen cells were thawed at room temperature and lysozyme was added. A detergent mix was then added. The viscosity was reduced by sonication and protein aggregates were harvested by centrifugation. The pellets were dissolved in a minimal volume of 4 M guanidine hydrochloride. The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein concentration was estimated by comparing the intensity of the coomassie blue-stained bands to a bovine serum albumin standard.

35

Example 2Nucleotide sequence of *S. uberis* CAMP factor gene

To obtain the nucleotide sequence of the *S. uberis* CAMP factor gene, each of the EcoRI, HindIII,
5 HincII and SacI fragments of pJLD21-2 was individually cloned into pTZ18R. Fragments were sequenced in both orientations as shown in Figure 1. The complete DNA sequence is presented in Figures 4A-4C (SEQ ID NO: ____). An open reading frame beginning with an ATG 10 codon located at positions 157 to 159 and terminating with the TAA stop codon at positions 925 to 927 was found which could encode a 256-residue polypeptide with a calculated molecular weight of 28,363. The ATG start codon is preceded by the purine-rich sequence 15 AAGAGG, which serves as a ribosome binding site in *E. coli* (Stormo et al. (1982) *Nucleic Acids Res.* 10:2971-2996).

Example 3Primer extension analysis

To localize the start site of transcription and the promoter region of the CAMP factor gene, primer extension analysis of RNA from *S. uberis* (ATCC 9927), *E. coli* JF1754(pJLD21) and *E. coli* 25 JF1754(pTZ18R) was done by using a synthetic oligonucleotide complementary to the DNA sequence from position 201 to 184. A strong primer extension product corresponding to base 91 ("T") was identified from both *S. uberis* and *E. coli* JF1754(pJLD21), but 30 not from *E. coli* JF1754(pTZ18R) (Figure 5). This data indicates that there is a major transcript of *S. uberis* CAMP factor gene initiated with an "A" residue (+1 in Figures 4A-4C; SEQ ID NO: ____). Both -10 and -35 regions, characteristic of *E. coli* promoters²⁹, were 35 identified at the upstream of the transcriptional start site (Figures 4A-4C; SEQ ID NO: ____).

Example 4Comparison of the *S. uberis* CAMP factor with
S. agalactiae CAMP factor

To compare the *S. uberis* CAMP factor with protein B of *S. agalactiae*, a concentrated culture supernatant of *S. agalactiae* containing protein B (Jürgens et al. (1985) *J. Chrom.* 348:363-370) was separated by SDS-PAGE and analyzed by immunoblotting with antibodies against the purified *S. uberis* CAMP factor. A 25 kDa protein band from the *S. agalactiae* supernatant (Figure 3A, lane 4) reacted in the immunoblot (Figure 3B, lane 4). This data indicated that monospecific antibodies raised against the *S. uberis* CAMP factor could cross-react with *S. agalactiae* protein B. This is not surprising since alignment of the 226-amino acid sequence of the *S. agalactiae* CAMP factor with the deduced 256 amino acids of the *S. uberis* CAMP factor showed 66.4% identical residues (Figure 6; SEQ ID NOS:__).

Example 5Distribution of CAMP factor genes in eight
S. uberis strains

To study the distribution of the CAMP factor gene in other *S. uberis* strains, chromosomal DNA prepared from eight *S. uberis* strains was digested with the restriction endonuclease *Hind*III and separated on an agarose gel. Southern blot analysis with the 576 bp *Hind*III-EcoRI fragment of pJLD21 as a probe (Figure 1) showed that a fragment identical in size to the *Hind*III fragment (1.2 kb) in pJLD21 was present in three *S. uberis* strains which were CAMP-reaction positive, while none of the CAMP reaction negative strains reacted with the probe (Figure 7). Thus, the CAMP-negative strains do not contain the cfu gene.

Example 6Immunogenicity and Protective Capability of
the CAMP Factor

S. *uberis* CAMP factor, encoded by pGH-CAMP,
5 was prepared from inclusion bodies as described in
Example 1. The antigen was formulated in VSA3
adjuvant which is a combination of Emulsigen Plus™
from MVP Laboratories, Ralston, Nebraska and
Dimethyldioctadecyl ammonium bromide (DDA) from Kodak
10 (Rochester, NY). The final concentration was 25 µg
per ml of CAMP factor, 30% Emulsigen Plus, 0.9% Tween-
80, and 2.5 mg per ml of DDA. The dose volume was 2
cc containing 50 µg of recombinant antigen.

Fifteen healthy lactating dairy cows from
15 the Pennsylvania State University Mastitis Research
Herd were used to study the ability of the S. *uberis*
CAMP factor to protect cows from mastitis. Animals
were assigned to two groups of five cows. Treatment
groups consisted of 1) experimental, given the vaccine
20 including the S. *uberis* CAMP factor, administered
intramuscularly at dry off and again 28 days later,
and 2) placebo (vehicle) administered via
intramuscular injection at dry off and again 28 days
later.

25 All animals were challenged in one quarter
with S. *uberis* on day four of lactation. Milk and
blood samples were obtained as outlined in Table 1.

30

35

TABLE 1
Sampling Schedule

	TIME	SAMPLE
5	dry off, D-0	serum, milk, immunization
	14 days dry, D+14	serum
	28 days dry, D+28	serum, immunization
	52 days dry, D+52	serum
10	calving, C-0	serum, milk, bacteriology
	4 days lactation, CH-0	serum, milk, bacteriology, challenge
	5 days lactation, CH+1	bacteriology
	6 days lactation, CH+2	bacteriology
15	7 days lactation, CH+3	serum, milk, bacteriology
	14 days lactation, CH+10	serum, milk, bacteriology
	21 days lactation, CH+17	serum, milk, bacteriology

20 The challenge strain of *S. uberis* (ATCC strain 9927) was obtained from a clinical case of bovine mastitis. The stock culture of *S. uberis* was grown in tryptic soy broth and individual aliquot were stored at -70°C on blood beads until needed. The bacterial challenge was prepared by rolling the stock bead cultures onto esculin blood agar plates containing 5% whole blood. After 24 hours incubation at 37°C, a single colony was used to inoculate 100 ml of Ultra High Temperature pasteurized (UHT) milk and incubated for 12 hours at 37°C. The 24 hour culture was mixed well and a 100 µl aliquot was removed to inoculate a second 100 ml of UHT milk. After a second 9 hour incubation at 37°C, the culture was serially diluted in 10-fold increments using sterile saline. The colony forming units (CFU) per ml of each dilution was determined by absorbance on a spectrophotometer and confirmed by plate pouring onto blood agar plates.

25

30

35

The dilution containing 200 CFR of *S. uberis* per ml of saline was selected for each challenge.

Total Ig titers for CAMP factor were determined by an indirect ELISA. Immunlon-2 plates 5 were coated with antigen in carbonate buffer. Prior to use, the plates were blocked with TBST (100 mM Tris Cl, pH 8.0; 150 mM NaCl; 0.05% Tween-20) and 3% BSA for 1 hour. After blocking, the plates were washed with distilled water. Serum and milk samples were 10 serially diluted in 3-fold increments using TBST containing 1% BSA. Rabbit antisera for *S. uberis* CAMP factor was also diluted and served as a positive control. Negative control samples contained TBST with 1% BSA. The diluted samples and controls were 15 transferred to the coated plates and were incubated for 1 hour at room temperature. The plates were washed thoroughly with distilled water and all wells were incubated with a horse radish peroxidase conjugate of goat anti-IgG diluted 1:2000 in TBST containing 1% BSA. Following a 1 hour incubation at 20 room temperature, the plates were washed with distilled water. The amount of antibody present in samples was visualized using ABT substrate. The titers of each sample were based on the absorbance 25 reading at 405 nm with a reference wavelength of 495 nm. A positive reading for samples was one in which the absorbance was two times the absorbance of the blank (negative control). Titers were determined by taking the reciprocal of the last dilution giving a 30 positive reading. Consistency among assay plates was monitored by the absorbance reading of positive controls.

The results are shown in TABLES 2 and 3. As can be seen, antibody titers were greater in the 35 vaccinated animals than in the placebo group.

TABLE 2
Average Serum Titers Following
Experimental Challenge with *S. uberis*

Treatment Group	Before Immunization	Before Challenge	After Challenge
Placebo A ¹	6.75	45.0	45.0
CAMP factor	3.00	819.00	445.50

5 ¹Serum titers of samples obtained from placebo
10 immunized animals screened for CAMP factor.

TABLE 3
Average Lacteal Antibody Titers Following
Experimental Challenge with *S. uberis*

Treatment Group	Before Immunization	Before Challenge	After Challenge
Placebo A ¹	0.00	0.00	1.33
CAMP factor	8.00	288.00	42.50

15 20 ¹Lacteal antibody titers of samples obtained from
placebo immunized animals screened for CAMP factor.

25 Somatic cell counts are a traditional
measure of mastitis in cows. Accordingly, milk was
assayed for somatic cells using standard assay.
Results are shown in TABLE 4. As is readily apparent,
immunized animals had a somatic cell count within
normal limits while the placebo group had cell counts
indicating the presence of mastitis. Thus, the CAMP
factor vaccine was effective in preventing mastitis.

30

35

TABLE 4
Average Milk Somatic Cell Counts
Following Experimental Challenge with *S. uberis*

Treatment Group	Before Challenge ¹	After Challenge ²
	1000 cells/ml milk	
Placebo A	130.50	2825.25
CAMP factor	251.75	51.50

10 ¹Milk SCC obtained from quarters immediately prior to intramammary challenge with *S. uberis*

²Milk SCC obtained from quarters 3 days following intramammary challenge with *S. uberis*

15 Thus, immunogenic CAMP factors are disclosed, as are methods of making and using the same. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

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CLAIMS

1. An isolated nucleic acid molecule comprising a coding sequence for an immunogenic 5 *Streptococcus uberis* CAMP factor.

2. The nucleic acid molecule of claim 1 wherein said coding sequence encodes an amino acid sequence substantially homologous and functionally 10 equivalent to the amino acid sequence shown at positions 1 through 256, inclusive, of Figures 4A-4C (SEQ ID NO:____), or an immunogenic fragment thereof.

3. The nucleic acid molecule of claim 1 15 wherein said coding sequence encodes an amino acid sequence substantially homologous and functionally equivalent to the amino acid sequence shown at positions 29 through 256, inclusive, of Figures 4A-4C (SEQ ID NO:____), or an immunogenic fragment thereof.

20 4. A recombinant vector comprising:
(a) a nucleic acid molecule according to any of claims 1-3; and
(b) control elements that are operably 25 linked to said nucleic acid molecule whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control elements is heterologous to said coding sequence.

30 5. A host cell transformed with the recombinant vector of claim 4.

6. A method of producing a recombinant CAMP 35 factor comprising:
(a) providing a population of host cells according to claim 5; and

(b) culturing said population of cells under conditions whereby the CAMP factor encoded by the coding sequence present in said recombinant vector is expressed.

5

7. A vaccine composition comprising a pharmaceutically acceptable vehicle and at least one immunogenic CAMP factor.

10

8. The vaccine composition of claim 7, wherein said CAMP factor is a *Streptococcus* CAMP factor.

15

9. The vaccine composition of claim 8, wherein said CAMP factor is a *Streptococcus uberis* CAMP factor.

20

10. The vaccine composition of claim 9, wherein said CAMP factor comprises an amino acid sequence substantially homologous and functionally equivalent to the amino acid sequence shown at positions 1 through 256, inclusive, of Figures 4A-4C (SEQ ID NO:____), or an immunogenic fragment thereof.

25

11. The vaccine composition of claim 9, wherein said CAMP factor comprises an amino acid sequence substantially homologous and functionally equivalent to the amino acid sequence shown at positions 29 through 256, inclusive, of Figures 4A-4C (SEQ ID NO:____), or an immunogenic fragment thereof.

30

12. The vaccine composition of any of claims 7-11, further comprising an adjuvant.

35

13. A method of treating or preventing a bacterial infection in a vertebrate subject comprising administering to said subject a therapeutically

effective amount of a vaccine composition according to any of claims 7-12.

5 14. A method of treating or preventing a streptococcal infection in a vertebrate subject comprising administering to said subject a therapeutically effective amount of a vaccine composition according to any of claims 7-12.

10 15. A method of treating or preventing mastitis in a mammal comprising administering to said mammal a therapeutically effective amount of a vaccine composition according to any of claims 7-12.

15 16. A method of producing a vaccine composition comprising:
 (a) providing at least one immunogenic CAMP factor; and
 (b) combining said CAMP factor with a pharmaceutically acceptable vehicle.

20 17. The method of claim 16, wherein said CAMP factor is a *Streptococcus* CAMP factor.

25 18. The method of claim 17 wherein said CAMP factor is a *Streptococcus uberis* CAMP factor.

30 19. The method of claim 18, wherein said CAMP factor comprises an amino acid sequence substantially homologous and functionally equivalent to the amino acid sequence shown at positions 1 through 256, inclusive, of Figures 4A-4C (SEQ ID NO:____), or an immunogenic fragment thereof.

35 20. The method of claim 18, wherein said CAMP factor comprises an amino acid sequence

substantially homologous and functionally equivalent to the amino acid sequence shown at positions 29 through 256, inclusive, of Figures 4A-4C (SEQ ID NO:), or an immunogenic fragment thereof.

5

10

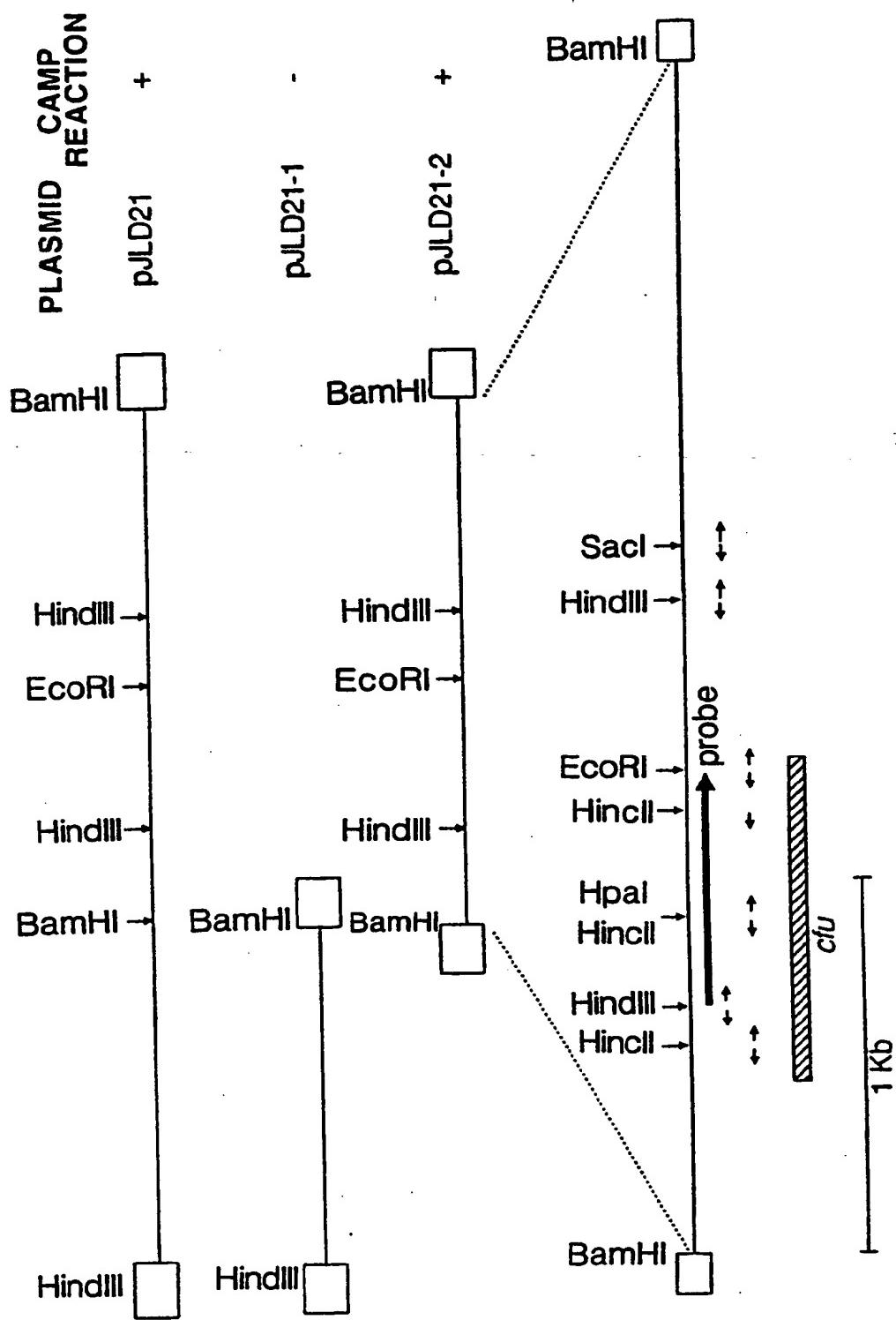
15

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25

30

35

**FIG. I****SUBSTITUTE SHEET (RULE 26)**

2 / 8

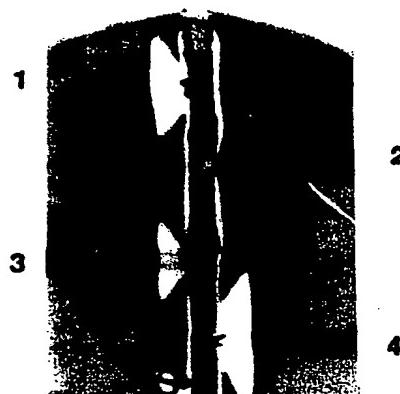


FIG.2

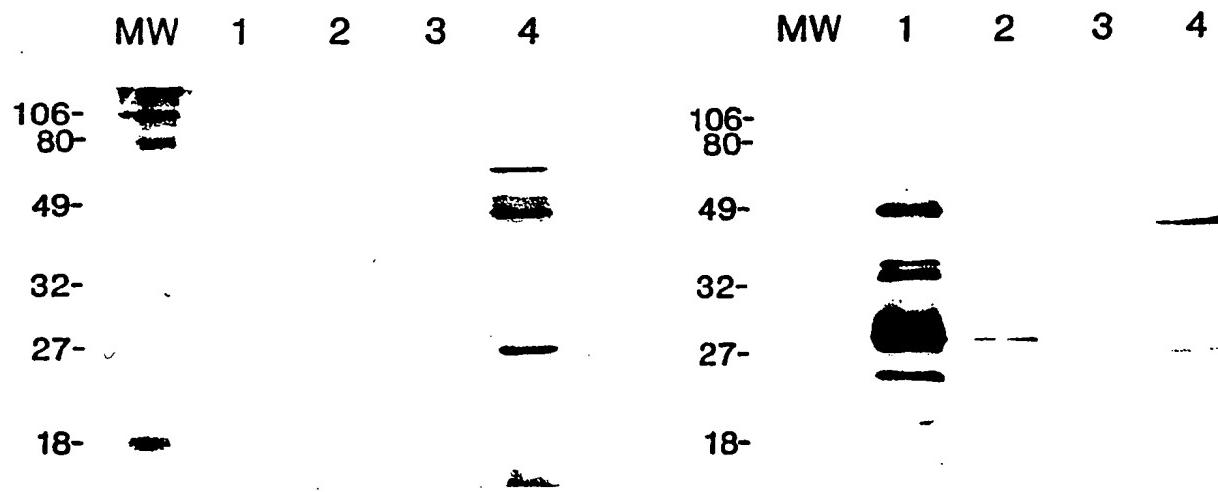


FIG.3A

FIG.3B

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3 / 8

ATGAAACATAAAAATTAAATTATAATTATTTATGATAATCACATATATTCAC
-35

60

⁺¹
TTAAAAAATTGTTACTGTATGATAAGGCATAAGGCTTATTTATGATAATGCAA
-10

120

TTTATAAACAATTATTTCAAAAGAGGAATGCTT ATG GAA TTC AAA AAG TTA CTT TAT
SD Met Glu Phe Lys Leu Tyr
a SIGNAL PEPTIDE a a >

TTA ACT GGT TCA ATC GCA GGA ATT ACT TTA TCC CCA ATT TTA ACA AGT GTC CAA GCA
Leu Thr Gly Ser Ile Ala Gly Ile Thr Leu Phe Ser Pro Ile Leu Thr Ser Val Gln Ala>
a a a a a a SIGNAL PEPTIDE a a a a a a >

AAT CAA ATA AAT GTT AGT CAA CCA TCT AAT GAA AGT ATT GAA CAG AAA AAA
Asn Gln Ile Asn Val Ser Gln Pro Ser Asn Asn Glu Ser Asn Val Ile Ser Gln Lys Lys>
b b b b b b MATURE PEPTIDE b b b b b b >

GAA GAA ATT GAT AAT AGT CTA AAT CAG GAA AGT GCT CAA CCA TAT GCC T^rGT AAA GAA GAT
Glu Glu Ile Asp Asn Ser Leu Asn Gln Leu Tyr Ala Gln Leu Lys Glu Asp>
b b b b b b MATURE PEPTIDE b b b b b b >

GTT AAA GGA ACT GAG AAA GAA CAA TCA GTT ATT TCA GCA ATT TCA GCT GTT GAA ATT TTA
Val Lys Gly Thr Glu Lys Glu Gln Ser Val Asn Ser Ala Ile Ser Ala Val Glu Asn Leu>
b b b b b b MATURE PEPTIDE b b b b b b >

FIG. 4A

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< / >

AAA ACT TCA CTT AGA GCT ATT CCT GAA ACA ATT TAT GAT TTA AAT TCG ATT GGA ACA AGA
 Lys Thr Ser Leu Arg Ala Asn Pro Glu Thr Ile Tyr Asp Leu Asn Ser Ile Gly Thr Arg >
b b b b b b b b MATURE PEPTIDE b b b b b b b >

GTA GAA GCA ATC TCT GAC GTG ATT CAA GCA ATT GTT TTT TCA ACG CAA CAG TTA ACA AAT
 Val Glu Ala Ile Ser Asp Val Ile Gln Ala Ile Val Phe Ser Thr Gln Gln Leu Thr Asn >
b b b b b b b MATURE PEPTIDE b b b b b b b >

AAA GTT GAT CAA GCT CAC ATT GAT ATG GGA TTT GCT ATT ACG AAA TTA CTT ATT CGC ATT
 Lys Val Asp Gln Ala His Ile Asp Met Gly Phe Ala Ile Thr Lys Leu Ile Arg Ile >
b b b b b b b MATURE PEPTIDE b b b b b b b >

GCA GAC CCA TTT GCT TCA AAT GAA TCC ATT AAA GGG CAA GTC GAA GCT GRT AAA CAA GTG
 Ala Asp Pro Phe Ala Ser Asn Glu Ser Ile Lys Gly Gln Val Glu Ala Val Lys Gln Val >
b b b b b b b MATURE PEPTIDE b b b b b b b >

CAA GCG ACT GTG CTT ACC TAT CCC GAT TTG CAG CCT ACG GAT AGA GCA ACT ATT TAC GTT
 Gln Ala Thr Val Leu Thr Tyr Pro Asp Leu Gln Pro Thr Asp Arg Ala Thr Ile Tyr Val >
b b b b b b b MATURE PEPTIDE b b b b b b b >

AAA TCA AAA TTA GAC AAG CTT ATT TGG CAA ACA AGA ATT ACC AGA GAT CAA AAA GTT CTT
 Lys Ser Lys Leu Asp Lys Leu Ile Trp Gln Thr Arg Ile Asp Gln Lys Val Leu >
b b b b b b b MATURE PEPTIDE b b b b b b b >

AAT GTA AAG AGT TTT GAA GTT TAT CAT CAA TTA AAT AAA GCT ATC ACA CAT GCA GTA GGT
 Asn Val Lys Ser Phe Glu Val Tyr His Gln Leu Asn Lys Ala Ile Thr His Ala Val Gly >
b b b b b b b MATURE PEPTIDE b b b b b b b >

FIG. 4B

5 / 8

GTA CAA TTA AAT CCA ACT GTA ACA GTT GCA CAA GTT GAC CAA GAA ATC AAA GTG CTA CAA 900
 Val Gln Leu Asn Pro Thr Val Thr Val Ala Gln Val Asp Gln Glu Ile Lys Val Leu Gln> 248
b b b b b b b b b b b b b b b b >

MATURE PEPTIDE _b_____b_____b_____b_____b_____b_____b_____b_____b_____b_____b_____b_____>

GAA GCA TTA AAT ACT GCT CTA CAG TAGGTAGAGATTGAATTGACGTATTAAAAGGACT 960
 Glu Ala Leu Asn Thr Ala Leu Gln> 256
b b b b b b b b b b b b >

MATURE PEPTIDE _b_____b_____b_____b_____b_____b_____b_____b_____b_____b_____b_____b_____>

GGAATTATTAAATTTCAGTCCTTTAGAATTTTTATTAGCTGATTACTTGTTGAAGAGA 1020

TTGGTGGAAATCAAGTACCATACTTCATTCTCCAAATACTTGTATGTCGATTCC 1080

CTTCCTAAACATAGCTAATTAGTTAGTTCTGGCTAACATGAAATTGACATGAATTGTT 1140

CAAATTACTAGGGTAAAGGTTTCTTTTATAAATTCATCATGACTAT 1190

FIG. 4C

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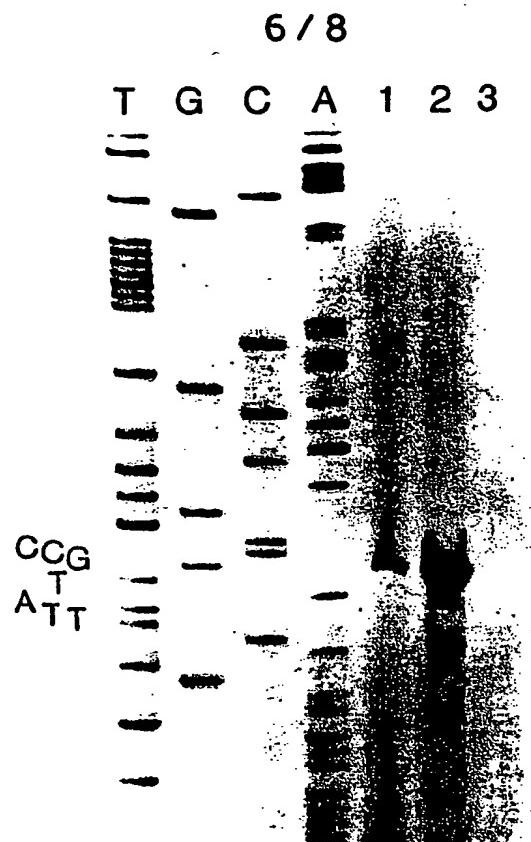


FIG. 5

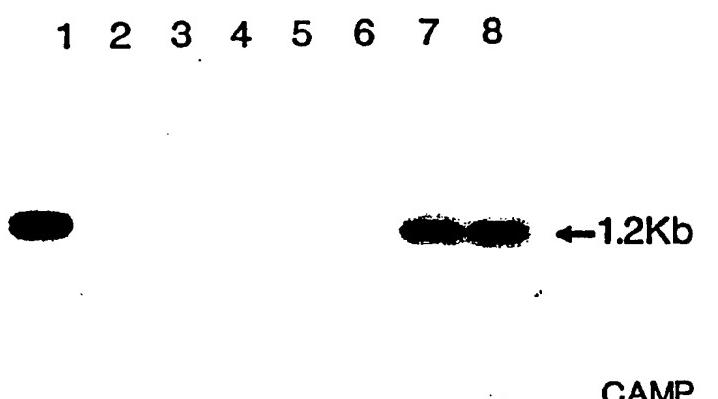


FIG. 7

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7 / 8

SUCAMP	- MEFKKLLYLITGSIAGITLFSPLTSVQANQINVSPQ-----SNNEENVIS -45
	: : : : :
SAGCAMP	- DQVTTTPQVNHVNSNNQAAQQMA -22
	: : : : :
SUCAMP	- QKKEEIDNSLNQESAOQLYALKEDVKGTEKEQSIVNSAISAVENLKTSLRAN -95
	: : : : : : : : :
SAGCAMP	- QKL-----DQDSIQLRNKDNVQGTDYEKPVNEAITSVEKLKTSLRAN -65
	: : : : : : : : :
SUCAMP	- PETIYDLNSIGTRVEAISDVIAIVFSTQQLTNKVDQAHIDMGFAITKLL -145
	: : : : : : : : :
SAGCAMP	- SETVYDLNSIGSRVEALTDVIEAITFSTQHLANKVSQANIDMGFGITKLV -115
	: : : : : : : : :
SUCAMP	- IRIADPPFASNESIKGQVEAVKQVQATVLTYPDLQPTDRATIVKSKE -195
	: : : : : : : : :
SAGCAMP	- IRILDPPFASVDSIKAQVNNDVKALEQKVLTYPDLKPTDRATIVTKSKLDKE -165
	: : : : : : : : :
SUCAMP	- IWQTRITRDQKVNLNKSFEVYHQLNKAITHAVGVQLNPVTVAQVQDQEIK -245
	: : : : : : : : :
SAGCAMP	- IWNTRFTRDKKVNLNKEFKVYNTLNKAITHAVGVQLNPNTVQQVQDQEIV -215
	: : : : : : : : :
SUCAMP	- VLQEALNTALQ -256
	: : : : : :
SAGCAMP	- TLQAALQTALK -226

FIG. 6

8 / 8

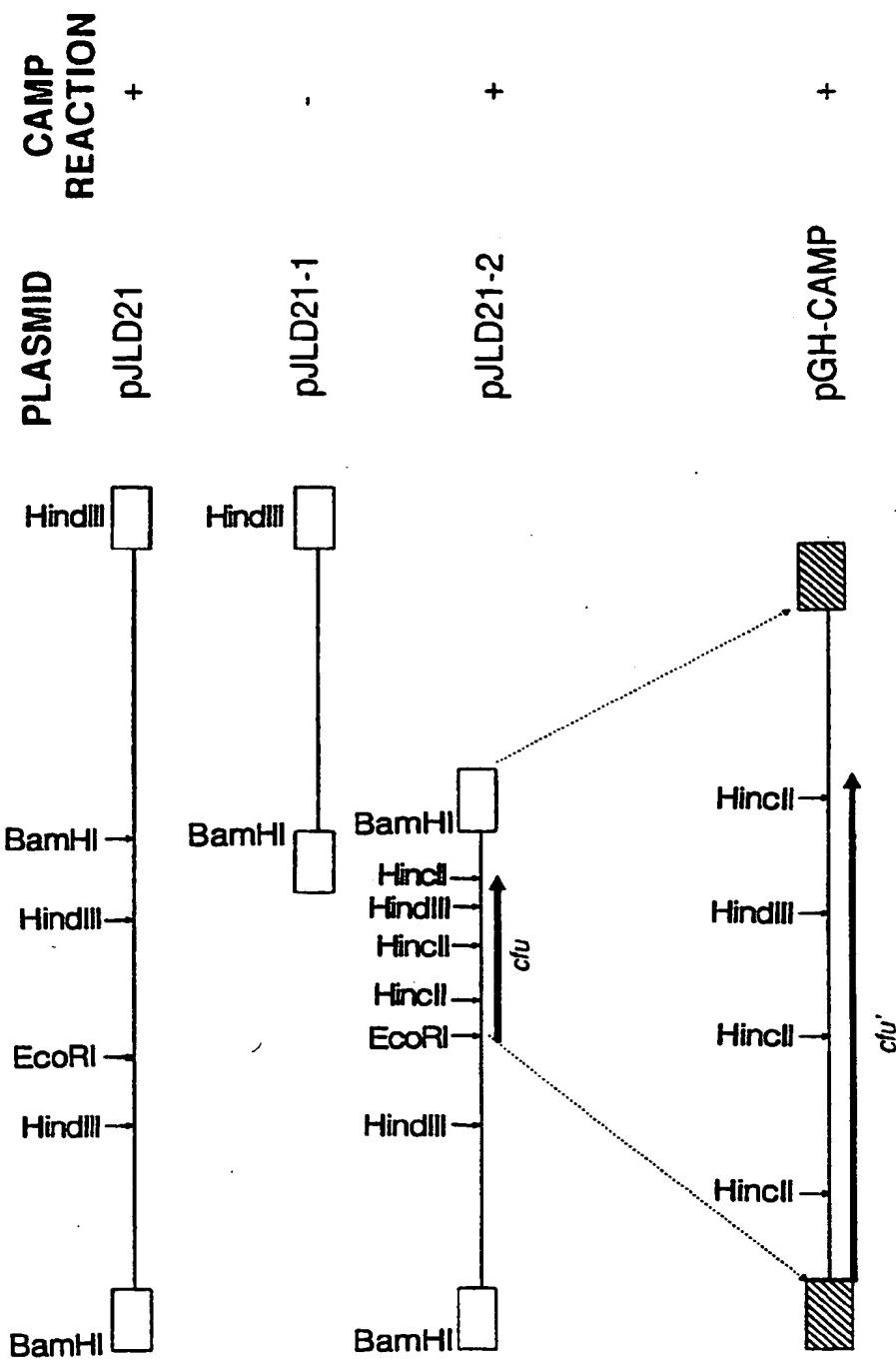


FIG. 8

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 96/00377

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/31 C12N15/74 C07K14/315 A61K39/09 C12N1/21
 //C12R1:46,C12N15:31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INFECT IMMUN, AUG 1988, 56 (8) P2174-9, UNITED STATES, XP002015858 SCHNEEWIND O ET AL: "Cloning and expression of the CAMP factor of group B streptococci in Escherichia coli." cited in the application see the whole document ---	1-12, 16-20
Y	EP,A,0 626 452 (AKZO NOBEL NV) 30 November 1994 see claims 1-11 ---	7-12, 16-20 -/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
17 October 1996	30.10.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Gurdjian, D

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 96/00377

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>ZENTRALBL BAKTERIOL [A], 1981, 249 (2) P190-4, GERMANY, WEST, XP002015859 SKALKA B ET AL: "Lethal effect of CAMP-factor and UBERIS-factor--a new finding about diffusible exosubstances of streptococcus agalactiae and Streptococcus uberis." see the whole document</p> <p>---</p>	1-6, 8-12, 17-20
A	<p>FEBS LETT, AUG 1 1988, 235 (1-2) P262-6, NETHERLANDS, XP002016186 RUHLMANN J ET AL: "Complete amino acid sequence of protein B."</p> <p>---</p>	1-6
A	<p>J EXP MED, MAR 1 1987, 165 (3) P720-32, UNITED STATES, XP002015860 JURGENS D ET AL: "Unspecific binding of group B streptococcal cecytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity." see the whole document</p> <p>---</p>	1-6, 8-12, 17-20
P,X	<p>MICROBIAL PATHOGENESIS, 20 (5). 1996. 297-307., XP002015861 JIANG M ET AL: "Cloning, sequencing and expression of the CAMP factor gene of Streptococcus uberis" see the whole document</p> <p>-----</p>	1-6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA96/00377

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 13-15 because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

(Information on patent family members)

International Application No

PCT/CA 96/00377

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0626452	30-11-94	CZ-A-	9401175	15-02-95